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Dopamine D2 Receptor Gene Expression in Human Adenohypophysial Adenomas

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The inhibitory effects of dopamine on adenohypophysial cells are mediated via dopamine subtype 2 receptor (D2R). Dopamine agonists inhibit hormone release and induce tumor shrinkage in most prolactin-secreting adenomas, whereas in other adenoma types such effects are sporadic. We investigated D2R gene expression by *in situ* hybridization (ISH) and immunocytochemistry in different types of pituitary adenomas. By ISH, a variable D2R signal was detected in 79 of 89 cases: 4 of 6 densely granulated and 8 of 8 sparsely granulated somatotroph, 4 of 4 mammosomatotroph, 7 of 7 mixed somatotroph-lactotroph, 4 of 4 acidophil stem cell, 16 of 16 sparsely granulated lactotroph, 11 of 16 corticotroph (functioning and silent), 3 of 4 silent subtype 3, 5 of 5 thyrotroph, 5 of 6 gonadotroph, 5 of 6 null cell, and 7 of 7 oncocytic adenomas. By immunocytochemistry, D2R protein was localized in cytoplasm and nuclei of 60 of 62 adenomas. In lactotroph adenomas, long-acting bromocriptine (BECLAR) induced a major increase in D2R mRNA, which was not accompanied by increased D2R immunoreactivity, suggesting mRNA stabilization. In conclusion, D2R gene is expressed in the majority of pituitary adenomas representing all tumor types. The significance of nuclear localization of D2R protein remains to be clarified.

Key Words: Bromocriptine; D2 receptor; immunocytochemistry; *in situ* hybridization; pituitary adenomas.

Introduction

Dopamine, a hypothalamic neurotransmitter, inhibits hormone secretion of adenohypophysial cells via dopamine subtype 2 receptor (D2R). Human and animal pituitaries contain high-affinity D2R bindings as demonstrated by radioreceptor assays *(1)*. Activation of lactotroph D2R results in suppression of prolactin (PRL) gene transcription, PRL synthesis, PRL release, and cell proliferation *(2,3)*. Based on these complex inhibitory effects of dopamine, different agonists have been extensively used for the treatment of patients harboring PRL-producing pituitary tumors. Despite the presence of D2R in other types of pituitary adenoma, administration of dopamine agonists has modest or no effect on tumor size and circulating hormone levels *(4,5)*. The causes of the poor response are obscure. With the progress made in molecular biology, deeper insights into the mechanisms of action and possible defects of D2R are just emerging.

D2R is a member of the G-protein-coupled receptor superfamily and is highly expressed in pituitary *(6,7)*. Dopamine binding to D2R produces multiple intracellular responses, such as inhibition of adenylyl cyclase activity, reduction of cytosolic Ca^{2+} levels, and activation of K^+ channel *(8)*. D2R mRNA is alternatively spliced by inclusion or exclusion of exon 6 to produce two isoforms of mRNA encoding proteins differing by 29 amino acids, called $D2_{444}$ (D2L) and $D2_{415}$ (D2S) receptors *(9,10)*. In human pituitary, both isoforms are found in equal amounts, whereas in rat pituitary the longer form is predominantly expressed *(10,11)*. Because the extra sequence of D2L is situated within a putative region that binds to G-protein, the isoforms may be important in determining the coupling to different G-proteins *(9,12)*.

In the rat pituitary, by *in situ* hybridization (ISH) D2R mRNA signal is very intense in intermediate lobe and much lighter and diffuse in the anterior lobe *(13–15)*. In human pituitary adenomas, D2R mRNA has been studied in growth hormone (GH) and PRL secreting as well as nonfunctioning ones by reverse transcriptase polymerase chain reaction *(16,17)* or ISH *(18–20)*. To our knowledge, the presence of D2R protein by immunocytochemistry has not been investigated in human pituitary and adenohypophysial tumors.

In the present study, we analyzed D2R gene expression in nontumorous human pituitaries and different types of adenomas including some exposed to bromocriptine, applying the methods of ISH and immunocytochemistry.

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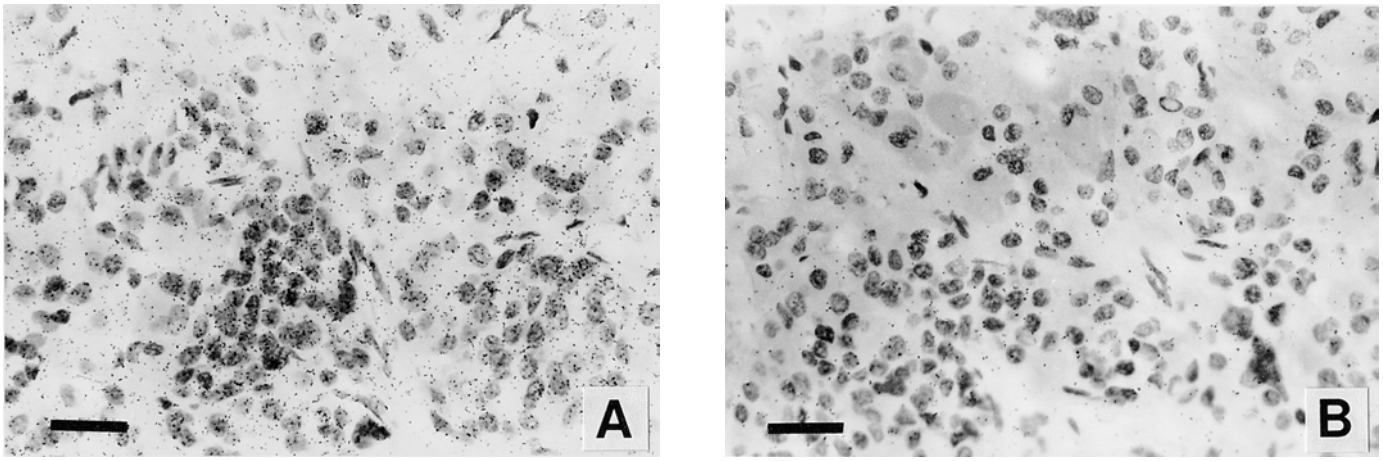

Fig. 1. (**A**) In a nontumorous adenohypophysis, the signal for D2R mRNA is present in most cells with variable intensity. (**B**) RNase pretreatment abolished almost completely the hybridization signal. Bar = 35 μm.

Table 1
ISH and Immunocytochemistry Results of D2R Gene Expression in Pituitary Adenomas[a]

	Hormone immunocytochemistry	D2R signal		D2R immunocytochemistry	
Type of adenoma	No. of positive cases[a]	Positive cases total no.	Intensity[b]	Positive cases total no.	Intensity[c]
Densely granulated somatotroph	GH:6; PRL:2; TSH:2; αSU:6	4/6	1	5/6	0–1/1
Sparsely granulated somatotroph	GH:8; PRL:5; TSH:4; αSU:3	8/8	1–2	6/6	1–2/1–2
Mammosomatotroph	GH:4; PRL:3; TSH:1; αSU:2	4/4	1–2	3/3	1–2/1
Mixed somatotroph-lactotroph	GH:7; PRL:7; TSH:2; αSU:5	7/7	2–3	3/3	1–2/1–3
Acidophil stem cell	PRL:4; GH:1; αSU:2	4/4	2	3/3	1–3/2–3
Sparsely granulated lactotroph	PRL:4	4/4	1–3	14/14	1–3/1–3
Sparsely granulated lactotroph (BEC-LAR)	PRL:6	6/6	>3	4/4	1–3/2–3
Sparsely granulated lactotroph (BEC)	PRL:6	6/6	1–3	NA	
Functioning corticotroph	ACTH:8; LH:5; αSU:4	5/8	1	5/6	1–3/0–2
Silent subtype 1	ACTH:4; LH:2; αSU:3	3/4	1–2	NA	
Silent subtype 2	ACTH:4; GH:1; LH:1; FSH:2; αSU:4	3/4	1–2	NA	
Silent 3	GH:1; PRL:2; TSH:3; FSH:1; αSU:2	3/4	1–2	NA	
Thyrotroph	TSH:4; GH:1; αSU:5	5/5	1–2	3/3	1–2/1–3
Gonadotroph	FSH:6; LH:6; αSU:5	5/6	1–2	5/5	1–2/1–2
Null cell	FSH:2; LH:2; αSU:4	5/6	1–2	3/3	1–3/1–3
Oncocytic	FSH:5; LH:5; αSU:5; PRL:2	7/8	1	5/5	1–2/0–2

[a]GH, growth hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.
[b]1 = 5–10 silver grains/cell; 2 = 10–15 silver grains/cell; 3 = 15–20 silver grains/cell; 4 = over 20 silver grains/cell.
[c]C = cytoplasmic; N = nuclear; 1 = weak immunostaining; 2 = moderate immunostaining; 3 = intense imunostaining; NA = not available.

Results

In Situ Hybridization

The nontumorous portions of pituitaries removed by surgery contained only adenohypophysial tissue. A detectable signal was obtained by exposing the sections to nuclear emulsion for 2 wk, whereas the tumors required only 1 wk of exposure. The signal for D2R mRNA was present in many cells including acidophils, basophils, and chromophobes. Some acidophils and basophils were devoid of silver grains (Fig. 1A,B).

The majority of adenohypophysial adenomas (88.7%) contained D2R mRNA with the probe detecting both isoforms (Table 1). D2R RNA was detected in all lactotroph adenomas. In untreated ones, the signal intensity varied from weak to intense (Fig. 2A,B). In nine sparsely granulated lactotroph adenomas removed from patients treated with BEC-LAR (long-acting repeatable injectable form), the intensity of hybridization signal was drastically increased (Table 2) (Fig. 2B). Because the silver grains were numerous and often coalesced, it was impossible to count them in every cell. The majority of these tumors responded

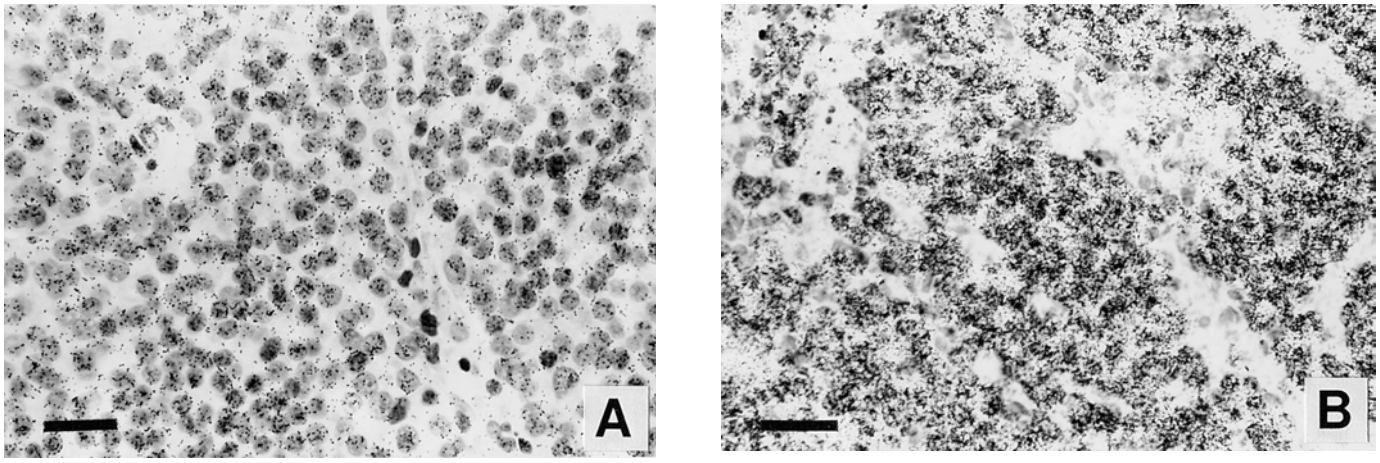

Fig. 2. (**A**) A sparsely granulated lactotroph adenoma shows a moderate hybridization signal for D2R mRNA. (**B**) A similar adenoma type removed from a patient treated with BEC-LAR exhibits a very intense signal for D2R mRNA. Bar = 35 μm.

Table 2
Summary of ISH Signal for D2R RNA in Lactotroph Adenomas from Patients Treated with BEC-LAR

Case no. sex, age	D2R mRNA (silver grains/cell)	BEC-LAR ± BEC	Blood PRL before/after BEC (μU/mL)[a]	Tumor shrinkage (MRI) (%)	Morphologic response
1. M 53	>60	1 × 100 mg	81,300/N	25–30	Unequal: medium and small cells
2. M 69	>80	2 × 100 mg	164,400/1839	30	Unequal: medium and small cells
3. M 53	>60	1 × 100 mg	17,500/N	30	Marked suppression
4. M 51	>60	1 × 100 mg	43,700/2930	<25	Marked suppression
5. F 58	>60	3 × 100 mg; +7.5 mg/d	39,500/N	25	Marked suppression
6. F 49	>60	1 × 100 mg	76,700/1709	10	Marked suppression
7. F 58	>40	1 × 100 mg	89,000/1229	25	Marked suppression
8. M 32	>40	3 × 100 mg	29,180/659	25	No response, most cells; some dark cells
9. M 28	>80	1 × 100 mg; +7.5 mg/d	157,000/343	10	Marked suppression

[a]N = normal (<500 μU/mL).

to the treatment by marked morphologic changes including heterochromatic nuclei, decreased cytoplasmic area, as well as involution of rough endoplasmic reticulum and Golgi zones. Normalization of blood PRL levels occurred in four patients, and all tumors showed 10–30% shrinkage according to magnetic resonance imaging (MRI) (Table 2). Six adenomas from patients receiving oral bromocriptine (BEC) up to surgery contained D2R signal similar in intensity to that found in lactotroph adenomas from untreated patients (Table 1).

D2R mRNA could not be detected in 2 of 6 densely granulated somatotroph, 3 of 8 functioning and 2 of 8 silent corticotroph, 1 of 4 silent subtype 3, 1 of 6 gonadotroph, and 1 of 6 null cell adenomas (Table 1). The hybridization signal showed a diffuse pattern with weak or moderate intensity (Fig. 3A,B).

Immunocytochemistry

In nontumorous pituitaries, D2R protein was localized in both the cytoplasm and nuclei of most cells (Fig. 4A,B). The intensity varied among cases, from weak to intense.

Most adenomas were immunoreactive for D2R protein. The immunoreactivity was present diffusely in the cytoplasm and in the nuclei of tumor cells. D2R immunoreactivity was weak in 10 lactotroph adenomas from untreated patients, and moderate to intense in 5 cases (Fig. 4C,D). In adenomas from patients treated with BEC-LAR, D2R immunostaining was also variable, from weak to intense in the cytoplasm, and more intense in the nuclei (Table 1, Fig. 4E). Most of the other types of adenoma presented weak cytoplasmic immunoreactivity and nuclear positivity in a variable number of nuclei (Fig. 4F–H). Scatter-plot analysis of intensities of hybridization signal for D2R mRNA

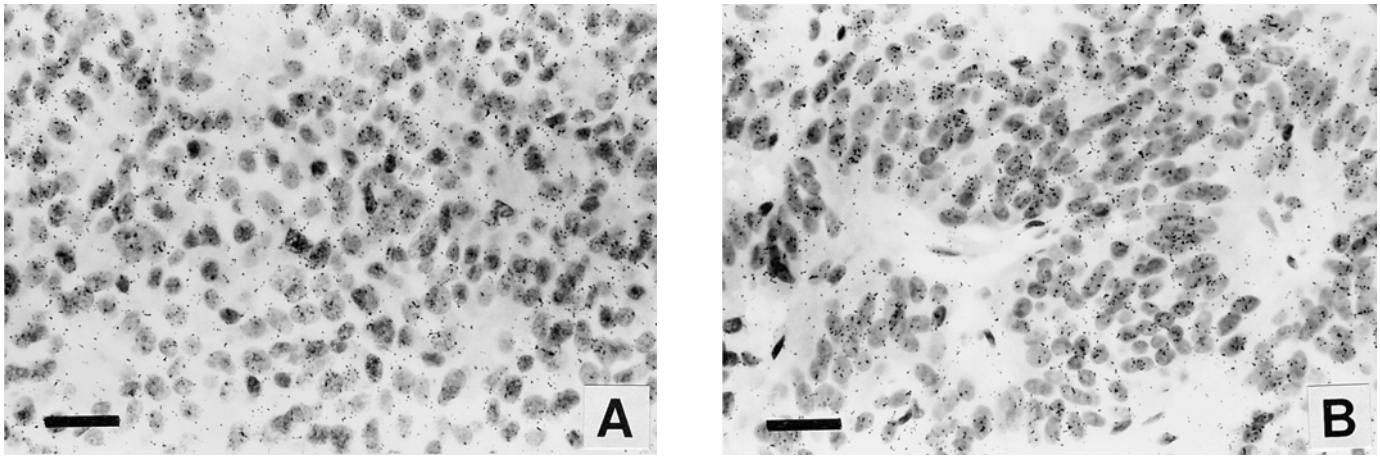

Fig. 3. (**A**) A functioning corticotroph adenoma and (**B**) a thyrotroph adenoma present a moderate to weak signal for D2R mRNA. Bar = 35 μm.

and D2R immunoreactivity revealed a poor correlation between the two variables ($R^2 = 0.14$) in pituitary adenomas.

Discussion

In nontumorous adenohypophyses, ISH demonstrated D2R mRNA in many cells including the acidophils, basophils, and chromophobes. The results are in agreement with those found in the rat pituitary, in which a diffuse distribution of silver grains was reported over the cells of anterior lobe *(13–15)*. Using immunocytochemistry to visualize haloperidol binding sites to dispersed rat pituitary cells identified ultrastructurally, dopamine receptors were localized in most lactotrophs, one third of somatotrophs, half of gonadotrophs, and a small proportion of corticotrophs and thyrotrophs *(21)*. In agreement with ISH results, D2R immunoreactivity was present in most cells. In contrast to D2R signal, which was weak, D2R immunoreactivity was intense in some specimens. The presence of D2R in various types of pituitary cells is not surprising. That dopamine inhibits other than PRL secretion was proven for GH and thyroid-stimulating hormone (TSH) by in vivo and in vitro studies *(22–25)*. Recent data indicate that mice lacking dopamine transporter have increased dopaminergic tone *(26)*. The homozygous mice are growth retarded and their pituitaries contain reduced numbers of GH- and PRL-immunoreactive cells.

Consistent with the presence of D2R transcripts in nontumorous adenohypophyses, the majority of pituitary adenomas representing all morphologic types contained the signal for D2R mRNA with a probe common for both receptor isoforms. The D2R mRNA signal was more abundant in adenomas than in nontumorous adenohypophyses, as deduced from the shorter exposure time to nuclear emulsion in the former ones. This was not the case for D2R immunopositivity, which differed among specimens from weak to intense. All sparsely granulated lactotroph adenomas contained D2R gene transcripts and encoded protein with some variations in intensity among cases. The dramatic increase in D2R mRNA found in lactotroph adenomas exposed to BEC-LAR was not accompanied by an increase in D2R cytoplasmic immunoreactivity. Only the nuclear positivity was in general stronger in BEC-LAR-exposed adenomas. In cultured normal rat pituitary cells, dopamine induced within 16 h a 400% increase in D2R mRNA *(27)*. In the GH_3 tumor cell line, which lacks high-affinity binding sites for dopamine but contains the D2R mRNA *(28)*, Johnston et al. *(27)* found no increase in signal after BEC treatment. A marked increase in D2R mRNA was found in all lactotroph adenomas exposed to BEC-LAR, regardless of the degree of responsiveness. It is possible that the conspicuous accumulation of D2R transcripts represents mRNA stabilization, since there is no proof that dopamine regulates the expression of its own receptor. In normal rat pituitary cells, the marked increase in D2R mRNA was associated with a delayed protein synthesis *(27)*. In adenomas exposed to oral BEC, D2R RNA signal was similar to that found in untreated ones. A moderate D2R signal was also reported in an atypical lactotroph adenoma from a patient who did not respond to oral BEC or CV-205-502 *(19)*, and D2R immunostaining proved to be negative (not included in the present study). It is conceivable that for the dramatic increase in D2R RNA, continuous exposure to BEC is necessary, because it occurs in patients treated with BEC-LAR.

The causes of heterogeneous response of lactotroph adenomas to BEC are unknown. Significantly decreased inhibition of adenylyl cyclase activity and reduced number of dopamine binding sites were found in some PRL-producing adenomas *(29)*. In BEC-resistant adenomas, Caccavelli et al. *(16)* reported a lower expression of short isoform (D2S) and of total D2R. In human BEC-resistant PRL-producing adenomas, $G_{\alpha i2}$ expression was decreased compared to responsive tumors whereas $G_{\alpha 0}$ protein was unchanged *(30)*.

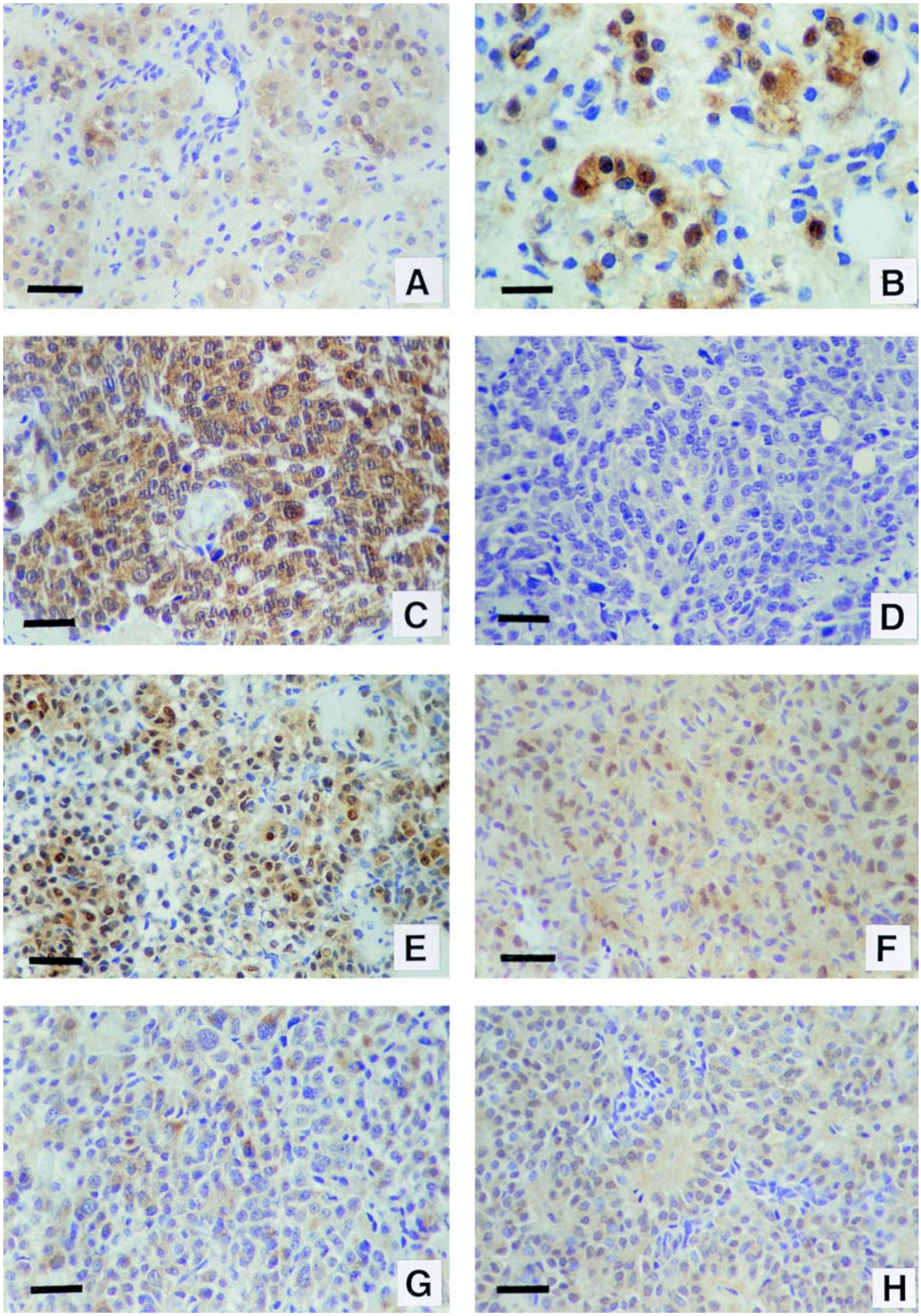

Fig. 4. Immunocytochemistry for D2R protein demonstrated in nontumorous adenohypophyses weak to moderate (**A**) or moderate to intense (**B**) cytoplasmic and nuclear immunostaining in many cells; (**C**) in a lactotroph adenoma a strong cytoplasmic and nuclear immunoreactivity that was abolished by preabsorption with D2R peptide (**D**); (**E**) in a lactotroph adenoma exposed to BEC-LAR, a moderate cytoplasmic and intense nuclear positivity; and a rather weak cytoplasmic and variable nuclear immunoreactivity in mammosomatotroph (**F**), corticotroph (**G**), and null-cell (H) adenomas. Bar = 50 (A,C–H) and 30 μm (B).

Estrogen-induced transplantable rat pituitary tumors have postreceptor defects *(31,32)* such as deficiency in $G_{\alpha 0}$ protein *(33)*. Targeted disruption of D2R gene in mice is associated with the development of pituitary lactotroph hyperplasia and tumors *(34,35)*. Screening studies in a large number of PRL- and GH-PRL-producing pituitary adenomas failed to demonstrate significant abnormalities of the coding region of D2R gene, despite its localization on the long arm of chromosome 11, which is predisposed to allelic loses *(36–38)*.

In accordance with previous investigations using different methodologies, most GH-producing adenomas expressed the D2R gene *(8,39,40)*. In acromegalic patients, dopamine agonist therapy lowered GH blood levels in approx 50% of them but did not change the size of tumors *(4,5)*. Spada et al. *(41)* reported a defective dopamine effect consisting of its failure to inhibit adenylyl cyclase in pituitary adenomas immunoreactive only for GH and without Gs-protein mutations. Mammosomatotroph adenomas cosecreting GH and PRL responded to dopamine with the typical D2R effector-coupling resulting in decreased adenylyl cyclase.

The effects of dopamine agonist therapy on other hormone-secreting or clinically nonfunctioning pituitary adenomas are scarce. Many but not all corticotroph adenomas associated with Cushing disease expressed D2R gene. Dopamine-binding sites were demonstrated in a corticotroph adenoma *(42)*. In some patients, administration of dopamine agonist decreased circulating adrenocorticotropic hormone (ACTH) levels *(43,44)*. BEC also inhibited ACTH secretion of a cultured human corticotroph adenoma *(45)*. Dopamine receptors were identified in some TSH-secreting adenomas *(46,47)*, and in a few patients with TSH-secreting adenomas, BEC lowered TSH blood level. However, in general, such tumors showed no response to dopamine agonist therapy *(48)*. Most gonadotroph adenomas expressed D2R gene, in accordance with previous reports showing the presence of dopamine-binding sites and lowering of gonadotropins and α-SU blood levels following administration of BEC *(49–51)*. A decrease in α-SU mRNA following dopamine treatment of an α-SU-secreting adenoma was found in vitro *(52)*. Most often, there is no change in tumor size.

The low signal for D2R mRNA in most null cell and oncocytic adenomas may explain the presence of a lower number of high-affinity binding sites in nonfunctioning adenomas *(42,49,53)*. Despite the presence of D2R, these tumors do not regress under dopamine agonist therapy. Based on in vitro studies, Spada et al. *(54)* suggested that nonfunctioning pituitary adenomas possess D2R defective transduction mechanism, since dopamine treatment reduced Ca^{2+} levels but had no effect on adenylyl cyclase activity.

The nuclear localization of D2R protein in both nontumorous and adenomatous pituitary is an unexpected finding. The BLAST search of GenBank of both nucleotide and amino acid sequence of the peptide against which the antibody was raised indicated no homology with any other sequence available. It can be speculated that the nuclear immunoreactivity is genuine, or that the peptide has high homology with a yet undiscovered protein. We tested the antibody in mouse pituitary and found the same cytoplasmic and nuclear localization of D2R protein. Moreover, the plasma membrane was immunostained as well, as expected for this transmembrane receptor.

The present study shows D2R gene expression with variable intensity in most types of pituitary adenoma, including all lactotroph adenomas. The weak signal in most non-PRL-producing adenomas and in some lactotroph adenomas may account for their poor or lack of response to dopamine agonist therapy. The negative results of ISH for D2R mRNA in a few tumors may be owing to the sensitivity of this technique applied on paraffin-embedded tissue or to the absence of D2R gene transcription. The massive accumulation of D2R mRNA in lactotroph adenomas exposed to BEC-LAR, which was not accompanied by an increase in D2R immunoreactivity, suggests mRNA stabilization rather than increased gene transcription. The significance of nuclear localization of D2R immunoreactivity remains to be studied.

Materials and Methods

Fragments of nontumorous adenohypophyses were obtained at surgery for corticotroph adenoma (five cases). For ISH study, adenohypophysial tumors surgically removed from 89 patients comprised 6 densely granulated somatotroph, 8 sparsely granulated somatotroph, 4 mammosomatotroph, 7 mixed somatotroph-lactotroph, 4 acidophil stem cell, 16 sparsely granulated lactotroph, 16 corticotroph (8 functioning, 4 silent subtype 1, and 4 silent subtype 2), 4 silent subtype 3, 5 thyrotroph, 6 gonadotroph, 6 null cell, and 7 oncocytic adenomas. The lactotroph adenomas were removed from six patients treated with oral BEC (2.5–7.5 mg/d, between 2 mo to 2 yr) up to surgery, nine patients treated with BEC-LAR (long-acting repeatable injectable form) (one to three injections of 100 mg/mo), and four untreated patients. Immunocytochemistry was performed in most cases subjected to ISH, except those for which no more tissue was available. Ten new cases of sparsely granulated lactotroph adenomas from untreated patients were included, as well (Table 1). The adenomas were previously diagnosed based on routine histologic examination, immunocytochemistry for adenohypophysial hormones, ultrastructural features, and clinical and biochemical data. For ISH, pieces of tissue fixed in neutral buffered formalin and embedded in paraffin were cut into 5-μm-thick sections. The oligonucleotide probe corresponding to human D2R complementary to a region common to both isoforms of receptor and corresponding to bases 1024–1064 (NEP-576) was purchased from DuPont Canada. The probe was labeled

by the 3'-end method with [^{35}S]dATPαS (deoxy-adenosine 5'-[α-thio]triphosphate) using a kit (NEP-100; DuPont Canada) and purified with the NENSORB-TM20 cartridge. The prehybridization and hybridization treatments were performed as described in detail elsewhere *(55)*. The concentration of probe was 4.5×10^5 cpm/slide. The exposure of sections to nuclear emulsion was 1 or 2 wk. As negative controls, RNase pretreatment and competition study with 100-fold excess of unlabeled probe were used. ISH was performed in two runs. To ensure that no variability from one batch to another was a confounding factor, five cases (two cases of normal adenohypophysis and three adenomas) served as internal control and were included in the second batch of slides.

Semiquantitative evaluation of the D2R mRNA signal intensity was performed by counting manually the number of silver grains with ×100 objective over an area covering a minimum of 30 nuclei of each case; a mean signal/cell was determined. Silver grains were also counted in RNase-pretreated slides to establish the background level of signal.

For D2R immunocytochemistry, the streptavidin-biotin peroxidase complex method was applied on deparaffinized, rehydrated sections. A goat polyclonal antibody against an epitope mapping at the amino terminus of human D2R protein (D2DR sc-7522; Santa Cruz Biotechnology) diluted 1:1000 was applied overnight at 4°C. Peroxidase activity was visualized with substrate and 3',3'-diaminobenzidine (DAB), followed by counterstaining with hematoxylin. For negative control, preabsorption of primary antibody with blocking peptide (sc7522 P; Santa Cruz Biotechnology) was performed.

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